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Non-invasive characterization of structure and morphology of silk fibroin biomaterials using non-linear microscopy

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Abstract

Designing biomaterial scaffolds remains a major challenge in tissue engineering. Key to this challenge is improved understanding of the relationships between the scaffold properties and its degradation kinetics, as well as the cell interactions and the promotion of new matrix deposition. Here we present the use of non-linear spectroscopic imaging as a non-invasive method to characterize not only morphological, but also structural aspects of silkworm silk fibroin-based biomaterials, relying entirely on endogenous optical contrast. We demonstrate that two photon excited fluorescence and second harmonic generation are sensitive to the hydration, overall β sheet content and molecular orientation of the sample. Thus, the functional content and high resolution afforded by these non-invasive approaches offer promise for identifying important connections between biomaterial design and functional engineered tissue development. The strategies described also have broader implications for understanding and tracking the remodeling of degradable biomaterials under dynamic conditions both *in vitro* and *in vivo*.
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1. Introduction

Design optimization of the biomaterials that are used as scaffolds onto which new or regenerated tissues are expected to grow is one of the major challenges facing tissue engineers. Numerous aspects of the biomaterials design, from the molecular structure and organization to the overall macro-architecture of these systems are known to impact significantly the development of new tissue *in vitro* and *in vivo* [1–4]. Scaffolds provide structural support and important environmental cues to cells that populate them, thus controlling to a large extent cellular proliferation [1,5], differentiation [6–8], deposition of new structural proteins [9,10] and ultimately the regeneration of functional tissue [4,11,12]. Traditionally, methods such as NMR, FTIR and X-ray spectroscopy have been used to assess biomaterials at the molecular level [13–17], while SEM and TEM have been invaluable tools for characterizing the three-dimensional morphology of biomaterial scaffolds [5,18–23]. Histology and immunostaining as well as assays for determining the expression levels of specific proteins are often used to assess how scaffolds interact with cells as tissues develop [5,10,18,20,22–24]. While all of these approaches provide sensitive and specific data, they are invasive. As a result, they reveal information about a single time-point along the development of a dynamically changing specimen. This limitation hinders full characterization and understanding of the relationships that exist between the structural, mechanical, architectural and biochemical properties of the scaffold and the corresponding properties of the developing tissue.

A number of optical methods have been developed to monitor non-invasively different tissue components in the context of disease diagnosis and monitoring [25–29]. Such methods

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have only recently started to be exploited as tools for assessing different properties of the cell and matrix components of engineered tissues [30–33]. The goal of this study was to determine the type of morphological and structural information that could be acquired about silk-based biomaterial scaffolds using spectral two photon excited fluorescence (TPEF) and second harmonic generation (SHG) imaging. This information will be essential in developing an optical biomarker toolkit that will allow us to monitor dynamically how such scaffolds interact with and are modified by cells as engineered tissues grow either *in vitro* or *in vivo*. Furthermore, these optical toolkits can also be extended to other biomaterial matrices as the systems are developed and optimized.

Silk is a natural protein polymer valued for its biocompatibility, light weight, and strength [34]. Processing methods for this polymer are well established for control of morphology, mechanical properties and environmental stability [35–38]. Due to these properties, silk is an excellent candidate for generating biomaterial scaffolds for engineered tissues. The *Bombyx mori* silkworm silk protein, fibroin, can be described by two structural models: Silk I, consisting of type II β turn, random coil domains, and mixed structures including alpha helices, and Silk II, consisting mostly of antiparallel β pleated sheets [14,15]. The β sheet content and the alignment of these β sheet crystals, along with the non-crystalline domains of the protein, are important determinants of the bulk mechanical properties and degradation kinetics of biomaterials generated from silk [14,36,37,39–41]. Most of the β sheet content and orientation of these crystalline domains is lost during the processing of silk fibroin into aqueous solutions, a step required for the regeneration of new biomaterial scaffolds for tissue culture [42]. The β sheet content and orientation can be reconstituted to different extents depending on the mode of material preparation [14,38,42]. Thus, the non-invasive, optical assessment of β sheet content and orientation of silk fibroin during biomaterial scaffold formation was one of the specific goals of this study.

Linear optical approaches, such as fluorescence and Raman spectroscopy have been used previously to characterize materials made from silk fibroin [39,43,44]. However, there are no studies to our knowledge on the non-linear optical properties of silk. Non-linear optical methods such as TPEF and SHG offer additional advantages for non-invasive imaging, including excitation in the near infra red region of the spectrum, where scattering is typically lower than in the visible region, and reduced photobleaching [45]. In TPEF and SHG, two photons, typically of the same energy, interact simultaneously with a molecule and yield either fluorescence emission (TPEF) or scattering (SHG) of a single photon. In the case of SHG, the scattered photon has the same energy as the collective energy of the two incident photons (i.e. there is no net energy loss and the wavelength of the scattered photon is at exactly half the wavelength of each one of the incident photons). In the case of TPEF, the wavelength of the incident photons is approximately twice as long as the wavelength of a photon required for linear excitation, while the fluorescently emitted photons have nearly identical spectral features as those resulting from single photon excitation. Because the probability of simultaneous interaction with two photons is orders of magnitude lower than single photon interactions, TPEF and SHG processes require the presence of high photon densities. As a result, these events are confined within a small volume in the apex of a focused cone of light and automatically yield optically sectioned, depth-resolved images. The confinement of the optical effect, and the use of low energy (longer wavelength) photons results in reduced thermal and photo damage within and outside the plane of focus [45]. Exploiting such processes in microscopic imaging platforms allows frequent sample assessment over long periods of time, without damage or contamination from elements outside the tissue culture environment.

2. Materials and methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

2.1. Preparation of silk fibroin

Cocoons of *B. mori* were kindly provided by M. Tsukada (Institute of Sericulture, Tsukuba, Japan). The cocoons were initially processed to remove the glue-like sericin proteins and to extract the pure silk fibroin component as previously described [39]. The resulting aqueous silk fibroin solution had an approximate concentration of 8% (wt) silk fibroin. For hexafluoro-2-propanol (HFIP) silk fibroin solutions, aqueous silk was lyophilized and redissolved in HFIP resulting in a 6% (w/v) fibroin solution.

2.2. Preparation of films and gels

Silk fibroin films were made by pipetting volumes of aqueous or HFIP silk solutions onto 35 mm glass bottom dishes (MatTek, Acton MA) and allowing the solvent to evaporate. Gels were made from aqueous solutions in 35 mm glass bottom dishes at silk concentrations of 8 and 4% with 6% (vol) 0.1 M HCl. The silk solutions were placed on the dishes first and the acid was titrated into the solution for a final volume of 2.5 mL. Dishes were then sealed with paraffin film and placed at 37 °C for 72 h [42]. β sheet content was induced in silk fibroin films by immersion in methanol for 30 min. Films were compressed and stretched by placing films in opposing clamps, applying pressure to secure the film and then drawing the clamps apart.

2.3. Preparation of scaffolds

Three-dimensional silk scaffolds were prepared as previously described [39]. The scaffolds were cut into discs (5 mm in diameter and 3 mm in thickness) dried at 60 °C, and autoclaved for further experiments. The pore size of the scaffolds was 550 ± 30 μm.

Human mesenchymal stem cells (Cambrex, East Rutherford NJ) were seeded onto an aqueous silk scaffold as described by Kim et al. [18]. The seeded scaffolds were placed on glass bottom dishes and imaged on day 21.

2.4. Two photon excited fluorescence and second harmonic generation microscopy

TPEF and SHG micrographs were acquired on a Leica DMIRE2 microscope with a TCS SP2 scanner (Wetzlar, Germany). The system was equipped with a 10× (NA 0.3) dry, 20× (NA 0.7) dry and a 63× (NA 1.2) water immersion objective. The excitation light source was a Mai Tai tunable (710–920 nm) titanium sapphire laser emitting 100 fs pulses at 80 MHz (Spectra Physics, Mountain View CA). Samples were placed on culture dishes with number 1.5 cover glass bottoms (MATTEK, Ashland MA) and excited at

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